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13. ABSTRACT (Maximum 200 Words)

In order to identify surface antigens that are differentially expressed in prostate cells during progression from normal to the cancerous and metastatic states, we are using antibody phage display and paired cell lines that differ in metastatic potential. In this method affinity subtraction and purification selects recombinant monoclonal antibodies binding to antigens that differ between closely related cell types, even without prior knowledge of the nature of the antigens involved. Research associates and students are being trained in a variety of techniques towards this goal. Collaborator Dr. Leland Chung at the Winship Cancer Institute developed lineage-related human prostate cancer cell lines, LNCaP and C4-2, that differ in metastatic potential. Using these cell lines and others, we have instituted procedures for selection and preliminary characterization of recombinant antibody scFvs. These include the maintenance of the human cell lines, their manipulation with inhibitors and biochemical and cytological analysis. Improved methods for induction, purification and labeling of bacterially expressed scFvs are concurrently being developed.

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INTRODUCTION:

The project involves the isolation of antibodies that identify molecules associated with the transition of human prostatic epithelium to metastatic adenocarcinoma. An immunochemical test for prostate specific antigen (PSA) has been valuable for early diagnosis of prostatic abnormalities including prostate adenocarcinoma (PCa). However, the PSA test does not distinguish benign from life-threatening disease, and no corresponding test for progression of the disease exists. In order to **identify surface antigens** that are differentially expressed in prostate cells during cancer progression we will use **antibody phage display** and paired cell lines that differ in **metastatic potential**. In this method, combining affinity subtraction with affinity purification selects MAbs that bind to antigens that differ between closely related cell types, even without prior knowledge of the nature of the antigens involved. Collaborator Dr. Leland Chung at the Winship Cancer Institute developed lineage-related human prostate cancer cell lines, LNCaP and C4-2B, that differ in metastatic potential [1] and by inference in the presence of metastasis-associated antigens. The specific aims of the research are to *1)* Isolate novel MAbs that bind to metastatic C4-2B cells more efficiently than to non-metastatic LNCaP cells; *2)* Evaluate the efficacy of the MAbs in inhibiting growth, migration, attachment and invasion by human prostate cancer cell lines; *3)* Correlate the presence and distribution of the antigens identified by the MAbs with patient specimens of known prognosis.

BODY:

Progress on items corresponding to the approved statement of work are addressed below:

Identify and hire personnel

Previous experience indicated that recruitment of postdoctoral associates to Clark Atlanta University is a difficult task. Consequently, rather than placing advertisements in scientific journals or at symposium job recruitment stations as had been done unsuccessfully in the past, contacts made with CAU and Emory University Collaborators and administration were exploited. An announcement placed on the Emory Medical School Office of Postdoctoral Education web site resulted in more than 20 applications. Eighty percent of these were from candidates actually in Asia; since our institutional capabilities to assist obtaining are minimal, these were not considered further. Ultimately, a former student in my lab (Dr. Tea Okou), and a former postdoctoral working with a CAU colleague (Dr. Jamil Haider) were introduced to the project in September 2002. Dr. Okou has prior experience with manipulation of recombinant antibodies and Dr. Haider had been recently working with fluorescence immunocytochemistry. A graduate student (Aron Tesfamichael), who had just completed his masters degree in physical chemistry, was recruited to the group in June 2002. A technician (Bernadette Jean-Joseph) with cell culture expertise worked under the auspices of this grant from August 2002- March 2003. A part-time administrative assistant (Su-wanna Barrow) facilitates management of the grant and coordination of Emory-CAU collaborative activities.

Former chair of the Chemistry Department and collaborator, Dr. Reynold Verret, left the University in Fall of 2003. Consequently his NIH/NIGMS (MBRS) project "Resistance to T-Cell Mediated Cytotoxcity", which ends in August 2003, is being supervised by Dr. Williams with a corresponding commitment in time. Although the scope of this project does not directly overlap with the USAMARC funded award, methods for immunological approaches to cancer detection, and in particular access to the Flow Cytometer, will enrich our ability to achieve the goals of "Biomarkers for Metastatic Prostate Cancer".

Establish meeting schedule with collaborating established investigator

Monthly meetings of the Emory Prostate Cancer SPORE Development Group has proved a convenient venue for informal and formal interaction with the collaborator of record on the proposal (Dr. Leland Chung), members of his group (particularly Drs. Chia-Ling Hsieh and Shian-Ying Sung), as well as establishment of new interactions. Dr. Williams and members of his group regularly attend seminars in the Winship's Elkin Cancer Biology Seminar Series, and have presented his research at the SPORE poster session and in the journal club of the Department of Obstetrics/Gynecology. Winship faculty engaged in prostate, breast and cervical cancer research have similarly presented seminars at CAU. In part as a result of the Collaborative Partnership Award, Dr. Williams has been appointed Adjunct Assistant Professor in the Emory University School of Medicine/Hematology Oncology Dept. This has afforded access to the online resources of the Emory Health Sciences Library, which has proved invaluable. Access to the Winship Microchemical and Proteomics Facility and the intellectual resource of its director, Dr. Jan Pohl, has allowed us to make strides towards developing affinity mass spectrometric methods of using the antibodies we will select to screen patient specimens (Specific aim 3).

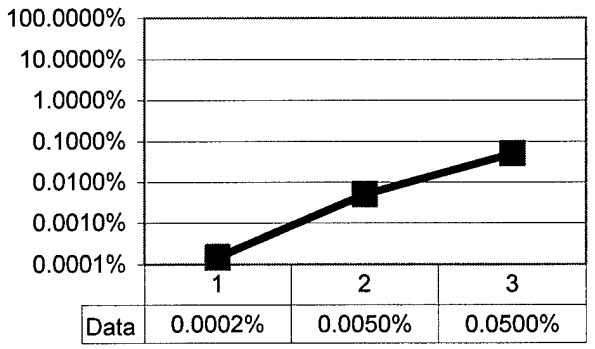
Collect cell culture material for selections

Although the cell lines were developed by Dr. Chung, rights to the lines remain property of The University of Texas M.D. Anderson Cancer Center and their representatives, the Urocor Corporation. We have obtained local IRB approvals for use of these line, completed materials transfer agreements with Urocor, and obtained and cultured the cell lines. Local IRB (CAU and Emory) for patient specimens is being modified from prior approvals dealing only with prostate tissues, to include serum samples. These specimens will not be examined until the middle of the upcoming fiscal year.

Subtraction and Selection on Cultured Cells

We have performed three rounds of subtraction and selection of the Tomlinson antibody scFv libraries [2] on LNCaP and C4-2B cells. The methods used for this selection have been informed by continued work on a related system which began in the lab in fall 2001. Cell line RWPE-1 is HPV-immortalized "normal" prostatic epithelium (non-tumorigenic) and line RWPE-2 is a *K-ras*-transformed (tumorigenic) derivative of RWPE-1 [3]. While the properties of these lines differ substantially from those of the LNCaP/C4-2B pair, this has proved a model for development of the methods of subtractive selection and clone analysis. We have used the Tomlinson I & J libraries in subtractive selections on glutaraldehyde-fixed RWPE-1 and RWPE-2 directly on cell culture plates. Preadsorbtion of the library on RWPE-1 cells, followed by selection on 1/3 to 1/10 the number of RWPE-2 cells, was performed for three rounds and resulted in an increase in the ratio of phage recovered to phage applied (Figure 1). This is typical of a good enrichment where specific binding clones are being selected. Posters describing the selection and a preliminary analysis of antigen binding were presented at the Cancer Research in Georgia Symposium and at the RCMI International Symposium on Health Disparities. However, subsequent analysis indicated that although cell-binding clones were selected, none demonstrated differential binding of the two cell lines. Thus, our revised protocol preadsorbs the library twice on 10-fold excess of subtractor cells (LNCaP) prior to affinity purification on selector cells. Fixation results in decreased reactivity of selected scFvs, and variably high incidence of background in immunoassays; LNCaP/C4-2 selections are therefore being performed with unfixed cell suspension. For detection of recombinant antibody (scFv) binding, use of the recommended anti-myc secondary antibody was shown to result in an artifact suggesting differential expression. Among alternate secondary reagents, Protein-L was found to minimize background and artifact. Of 48 clones from the 3rd round of panning screened by cell-ELISA using *E. coli* periplasmic extracts and Protein-L-peroxidase, 13 shows substantial binding to RWPE-1 and RWPE-2 cells. Our results further indicate that cell-ELISA tends to be more variable than ELISA performed against total membrane extracts of mammalian cells. Consequently, selected clones in the future will be screened by membrane-ELISA.

Figure 1: Proportion of phage bound to RWPE-2 cells



We spent several months trying to characterize 8 scFv clones which we believed bound preferentially to RWPE-2 cells. DNA sequence of these clones indicates that they are all different both genetically and in the deduced amino sequence of the antigen combining sites. Surprisingly all clones are derived from one of the two libraries (I), and all had at least one amber stop codon. This is permissible since the *E. coli* strain (XL1Blue [4]) used generating phage for selection carries an amber suppressor. We calculated that ~ 56% of clones from library I (18 residues x NNS) theoretically carry at least one amber codon; the apparent enriched frequency

among the selected clones suggests that there is a selective advantage for stop codons; in fact we and others have observed some toxicity to bacteria expressing high levels of exported scFvs [5;6]. We have thus modified our protocol for growth of expression libraries to include glucose repression; this will limit the induction of the recombinant protein even when scFv-phage are being produced, and should reduce the selective bias for proteins that are expressed at lower levels. Yield of soluble scFv is correspondingly lower than anticipated; IMAC purification of a control scFv indicates about 180μg/L bacterial culture. We are currently optimizing protocols that may enhance production of soluble scFv by more than 20-fold [7]. This will be necessary in order for us to obtain substantial amounts of pure scFv to test the biological activity of antibodies we will identify (Aim 2) as well as for direct scFv labeling (Aim 3). Similarly, low-cost IMAC matrices for large scale scFv purification [8] will be synthesized in collaboration with polymer chemists at CAU.

Immunocytochemistry/Survey of tissue specimens

Immunocytochemical analysis for binding of scFvs to cultured PCca cells is largely in agreement with cell-ELISA data; however colorimetric peroxidase staining was found to provide enhanced sensitivity over immunofluorescence. Two of the available microscopes belonged to Dr. Verret who will take them with him; an inverted microscope has thus been purchased and a high-resolution (rather than high-sensitivity) digital camera is being ordered to permit better quantitative analysis of stained cells. Immunochemical analysis of cultured cells is important in preparation for analysis of biopsy specimens; in addition we have observed unexpected differences in the level of epidermal growth factor receptor (EGFR) between RWPE-1 and RWPE-2.

One of the strengths of our subtractive selection approach is that it permits the detection of any antigen, whether of protein or non-protein nature; typical proteomic analysis does permit analysis of carbohydrate antigens. Towards distinguishing cell-surface carbohydrate antigens, we have extracted acidic glycolipids (gangliosides) from the cell lines. Increased prevalence of ganglioside GM_3 in RWPE-1 and decreased prevalence of another glycolipid that is slow migrating in TLC in RWPE-2, have been observed. This result contrasts with published reports suggesting that *Ras* activation results in increased expression of GM_3 in murine fibroblasts[9]. We have established, furthermore, that selected scFv10 does not recognize a ganglioside antigen.

In anticipation of obtaining scFv clones that distinguish lineage related cell lines, we have been examining the effect of *in vitro* growth conditions that would be expected to modulate known differences. Posttranslational prenylation of proteins, either farnesylation or geranylgeranylation, is required for the transforming activity of *K-ras*, the cause of tumorigenicity in RWPE-2. Treatment of RWPE-2 with a combination of farnesyl transferase inhibitor FTI-276 and geranylgeranyl transferase inhibitor GGTI-298 [10] causes a decrease in anchorage independent growth. Surprisingly, GGTI-298 appears to increase anchorage independent growth in RWPE-1, implying that a geranylgeranylated protein is involved in negative growth regulation in this cell line. A poster describing our observations will be presented at the meeting of the International Union of Biochemistry and Molecular Biology in July 2003. Although *K-ras* is not mutant in LNCaP cells [11], this has not been determined for its C4-2B derivative, and the effect of inhibitors will be examined. Analysis of the regulation of

identified antigens by this kind of approach will be an important part of secondary antigen characterization and understanding their biological and oncologic significance (Aim 2).

KEY RESEARCH ACCOMPLISHMENTS:

- PI appointed Adjunct Assistant Professor of Hematology/Oncology, Emory University Medical School
- Resolved problems in immunochemical detection of scFv on human cell lines
- Obtained paired cell lines and began subtractive selections
- Determined differences in ganglioside and EGFR expression in cells lines
- Identified and addressed difficulties associated with bacterial expression and purification of recombinant antibodies.
- Obtained preliminary evidence that prenylation inhibitors proposed for cancer therapies may have unexpected effects

REPORTABLE OUTCOMES:

Poster Presentations

Flowe, CF* and Williams MNV. "Recombinant Antibodies Specific For Cell Surface Antigens Of Tumorigenic Prostate Cells" Biomedical and Health Sciences Institute: Cancer Research in Georgia Symposium at Athens on June 2-3

Williams MNV, Flowe CM*, Haider J. "Identification of Tumor-Specific Antigens of Prostate Cells using Antibody Phage Display". Eight RCMI International Symposium on Health Disparities, December 8 - 11, 2002, Honolulu, Hawaii

Williams MNV, Haider J, Jean-Joseph B, DeLeon-Mancia M, and Flowe C. "*K-ras* Regulation Of Anchorage-Independence And Antigen Expression In Human Prostate Cell Culture". International Congress of Biochemistry and Molecular Biology. July 20-24, 2003

CONCLUSIONS:

Personnel and resources have been put in place to facilitate the proposed goals of the research project. The work performed to date has resulted in a substantial number of improvements in the procedures to be used for selection and characterization of scFv that distinguish metastatic from non-metastatic cell lines. We have also begun the development of novel analytical methods, a "mass spectrometric immunoassay" that we anticipate will permit identification of the distinguishing antigens, and may be eventually developed for routine clinical screening of patient specimens.

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APPENDICES: None